Subterranean Biology 55: 187–215 (2026)
doi: 10.3897/subtbiol.55.185185
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

An updated phylogeny of the family Stenasellidae (Crustacea, Isopoda), with a new species of *Stenasellus* from southern Vietnam

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Academic editor: Fabio Stoch | Received 15 January 2026 | Accepted 21 March 2026 | Published 15 April 2026

https://zoobank.org/8F6D2242-2FE4-49A4-949C-56A75C39DA6F

Citation: Marin IN, Palatov DM, Văn Thinh N, Deharveng L, Konecny-Dupré L, Douady CJ, Malard F (2026) An updated phylogeny of the family Stenasellidae (Crustacea, Isopoda), with a new species of *Stenasellus* from southern Vietnam. Subterranean Biology 55: 187-215. https://doi.org/10.3897/subtbiol.55.185185

Abstract

Our current understanding of the molecular systematics of Stenasellidae, a species-rich family of obligate groundwater isopods known from Africa, Asia, Europe and North-America, is based primarily on specimens of the genus *Stenasellus* Dollfus, 1897 collected in Europe. Here, we provide an updated phylogeny of the family comprising specimens of *Stenasellus* collected from multiple countries in Asia, including Iran, Turkmenistan, India, Cambodia, and Vietnam as well as specimens of *Metastenasellus* Magniez, 1979 collected in Africa. First, we used COI-based molecular species delimitation methods to delimit putative species within the family Stenasellidae. Second, we built a phylogeny of the Stenasellidae comprising 48 putative species, for which two mitochondrial and two nuclear genes were available. Third, we describe *Stenasellus angiangensis* **sp. nov.** from the hyporheic zone of streams located in mountainous islands of the Mekong River Delta, the first representative of the family Stenasellidae described from Vietnam. We show that the number of putative species delimited using various COI-based molecular species delimitation methods largely surpass the number of species described based on morphological criteria. The four-gene phylogeny reveals, for the

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first time, a robustly supported and broadly distributed Asian clade comprising *Stenasellus* species from Iran, India, Cambodia, and Vietnam, including *Stenasellus angiangensis* **sp. nov.** as the sister taxon to *Stenasellus cambodianus* Boutin & Magniez, 1985 from southern Cambodia. Our phylogeny strongly questions the monophyly of the genus *Stenasellus*, even though the latter cannot be definitively rejected due to difficulties in resolving the phylogenetic relationships among the clades identified within stenasellids.

Keywords
Asellota, Asia, Diversity, groundwater, Indochina, Peracarida, subterranean

Introduction

Groundwater systems constitute the largest freshwater habitat on Earth and represent a significant, albeit underappreciated, component of global freshwater biodiversity (Malard 2022). Among the taxa inhabiting groundwater, crustaceans are particularly diverse: in fact, a greater number of crustacean species have been documented in groundwater than in surface freshwater habitats in Europe (Stoch and Galassi 2010). Despite this, the diversity inventory in groundwater habitats remains far from complete, owing to the inherent challenges of sampling in these habitats. Groundwater organisms are often difficult to collect, occur at low densities, and typically exhibit highly restricted geographic distribution (Ficetola et al. 2019; Mammola et al. 2024). Furthermore, knowledge of groundwater biodiversity is unevenly distributed across continents, with most studies concentrated in Europe, North America, and Australia, while relatively few have been conducted in Africa, South America and Asia (Brancelj et al. 2013; Zagmajster et al. 2018, 2023).

The family Stenasellidae currently comprises 72 described species distributed in 10 genera (Jugovic et al. 2024; Boyko et al. 2026). All stenasellids are eyeless, depigmented and inhabit exclusively subterranean freshwater environments. The geographic range of the family extends longitudinally from Mexico to Borneo (Indonesia) and latitudinally from Java (Indonesia) to Slovenia (Jugovic et al. 2024) (see Fig. 1A). The discovery and taxonomic description of stenasellids in Asia are more recent compared to Europe, North America and Africa. By 1980, most of the species currently known on the latter three continents had been described, whereas only a single species had been described from Asia. Today, Asia counts 22 species of Stenasellidae, all currently assigned to the genus *Stenasellus* Dollfus, 1897. This is more than the 16 species known from Europe, and new species are regularly being described from Asia (Bakhshi et al. 2018; Khalaji-Pirbalouty et al. 2018; Messana et al. 2019; Jugovic et al. 2024).

Molecular systematics of Stenasellidae began in the early 2000s but progress has been limited, primarily due to the relatively old available museum collections, which are poorly suitable for molecular studies, as well as difficulty of re-collecting topotypic specimens from localities across the entire distribution range of the family (Ketmaier et al. 1999, 2003; Morvan et al. 2013; Pountougnigni et al. 2021; Lagnika et al. 2024).

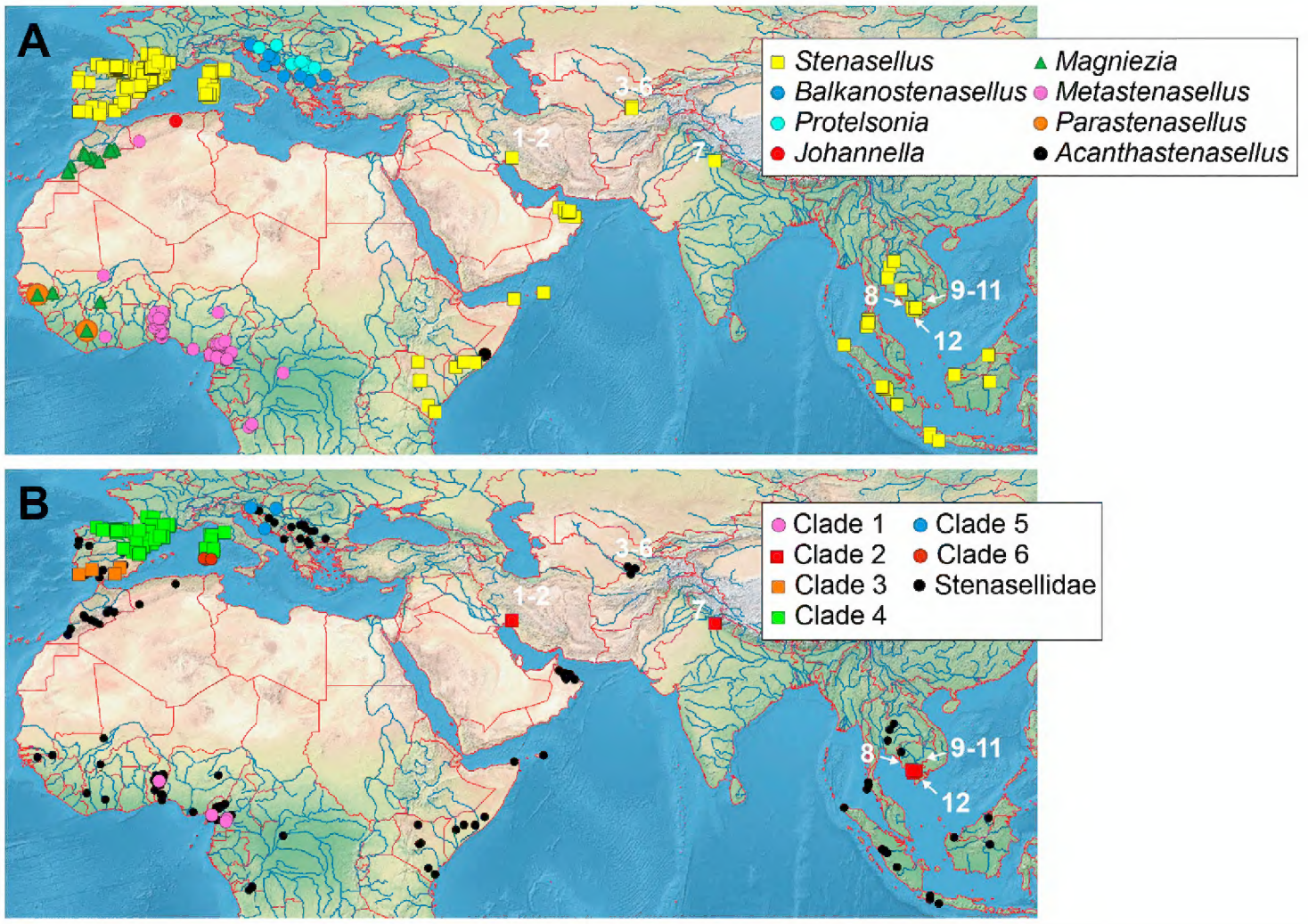

Figure 1. A occurrence of genera of Stenasellidae Dudich, 1924 in Europe, Africa and Asia (occurrence data extracted from the World Asellidae Database, Saclier et al. 2024) **B** distribution of the six polytypic well-supported clades of Stenasellidae as identified in the four-gene phyogeny (Fig. 2). Numbers 1 to 12 correspond to sites in Asia, where specimens were collected as part of the present study.

The latest multi-gene phylogeny of the Stenasellidae included 40 terminal leaves, corresponding to 35 species of *Stenasellus*, one species of *Balkanostenasellus* (Cvetkov, 1967) and one species of *Protelsonia* Méhely, 1924, all from Europe, two species of *Stenasellus* from Iran, and one species of *Magniezia* Lanza, 1966 from Morocco. Consequently, our current understanding of the molecular systematics of Stenasellidae is based primarily on specimens of the genus *Stenasellus* collected in Europe, despite that genus has an Afro-Eurasian distribution (Fig. 1A).

In the present study, we provide an updated phylogeny of Stenasellidae that comprises specimens of *Stenasellus* collected from multiple countries in Asia, including Iran, Turkmenistan, India, Cambodia, and Vietnam as well as specimens of *Metastenasellus* Magniez, 1979 collected in Africa, including Benin and Cameroon. First, we used molecular species delimitation methods performed on 137 haplotypes of the partial mitochondrial cytochrome oxidase subunit I (COI) to delimit molecular operational units (MOTUs) within the family Stenasellidae. Second, we built an updated phylogeny of the Stenasellidae comprising 48 MOTUs, using two mitochondrial and nuclear genes. Third, we describe a new species of *Stenasellus* from Vietnam using morphological and molecular criteria.

Material and methods

Phylogenetic analyses and molecular taxonomy

Sampling of *Stenasellus* from Asia

We obtained specimens of *Stenasellus* from 12 locations in Asia (Fig. 1). The *Stenasellus* specimens from Iran (sites 1–2 in Fig. 1) and Turkmenistan (3–6) were sampled by collaborators, whereas specimens from India (7), Cambodia (8) and Vietnam (9–11, 12) were sampled by the authors. Stenasellids were collected by filtering or pumping the water of caves, springs and the hyporheic zone of streams. All samples were preserved in 90% ethanol for further DNA analysis. In the laboratory, the collected specimens were identified mostly using the morphology of the male pleopods I and II, as well as some features of the pleopods III–V and the uropods (Magniez 1991). We provide below the list of species, sampling locations, and the material examined from Asia.

Stenasellus tashanicus Khalaji-Pirbalouty, Fatemi, Malek-Hosseini & Kuntner, 2018. Iran: Khuzestan Province: Sarjooshar, 30°51'54.2"N, 50°10'29.0"E, in Tashan Cave (site number and code: 1, TASHANCA), coll. Y. Fatemi, 27 August 2016, 1♂, 3♀♀, 3 juveniles (UCBLZ: Coll Number 2012.11.25.79).

Stenasellus stygopersicus Jugovic, Malek-Hosseini & Issartel, 2024. Iran: Khuzestan Province: Sarjowshar, 30°52'17.7"N, 50°10'06.6"E, in Chah Kabootari Cave (2, CHAHKABO), coll. M.J. Malek-Hosseini, 17 October 2017, 10♀♀, 6♂♂ (UCBLZ: Coll Number 2012.11.24.80 to 2012.11.24.98; see Jugovic et al. 2024).

Stenasellus asiaticus Birstein & Starostin, 1949. Turkmenistan: Lebap Province: Karlyuk, 37°31'17.6"N, 66°21'14.3"E, in Akbulak spring (3, AKBULASP), coll. J. Grego, J. Lips & B. Lips, 21 April 2024; 3♀♀, 2♂♂ (UCBLZ: Coll Number 2012.11.25.80); Kundzhak, 37°32'16.7"N, 66°24'20.8"E, in thermal karst spring Kainar (4, KAINARSP), same collectors, 16 April 2024; 1♀♀, 11 juveniles (UCBLZ: Coll Number 2012.11.25.81); Koyten, 37°49'41.2"N, 66°24'37.6"E, in Kaptarhana Cave (6, KAPTARHA), same collectors, 11 April 2024; 2 juveniles (UCBLZ: Coll Number 2012.11.25.82); Karabulak, 37°36'19.8"N, 66°21'11.8"E, in Karabulakh spring (5, KARABULA), same collectors, 16 April 2024; 1♀, 3 juveniles (UCBLZ: Coll Number 2012.11.25.83).

Stenasellus sp. India: Uttarakhand Province: Uttarakhand, 30°25'40.7"N, 77°43'20.8"E, in the hyporheic zone of the Assan River (7, ASSANGU), coll. C.J. Douady & F. Mermillod-Blondin, 7 December 2022, 1 juvenile ♂, 2 juveniles (UCBLZ: Coll Number 2012.11.25.84).

Stenasellus cambodianus Boutin & Magniez, 1985. Cambodia: Kampong Trach Province: Kampong, 10°34'51.9"N, 104°28'16.9"E, in Kampong Trach water cave (8, KAMPONGC), coll. A. Bedos & L. Deharveng, 25 November 2005, 2♀♀ (UCBLZ: Coll Number 2012.11.25.85). Vietnam: Kiên Giang Province, Binh An, 10°8'12.9"N, 104°38'35.1"E, in Hang Giếng Tiên (Cave) (12, HAGITICA), coll. L. Deharveng & M. Lukic, 8 October 2014, 3♂♂ UCBLZ: Coll Number 2012.11.25.86).

Stenasellus angiangensis sp. nov. Vietnam. Location of sites (sites 9–11, Fig. 1) and material examined are provided in the species description section of that article. The type material of the new species from Vietnam is deposited at the collection of the Zoological Museum of Moscow State University, Moscow (**ZMMU**). Additional material is deposited in personal authors' collection (IM and DP) in the A.N. Severtsov Institute of Ecology and Evolution of RAS, Moscow, Russia (**LEMMI**), in the Laboratory of Hydrobiology of the Southern Branch of the Joint Vietnam–Russia Tropical Science and Technology Research Center, Ho Chi Minh City, Vietnam, and in the zoological collection (CD and FM) of the University Claude Bernard of Lyon, France (**UCBLZ**, https://ucblz.univ-lyon1.fr/).

Molecular data collection

We refer the reader to Saclier et al. (2024) for details about methods used for DNA extraction, gene fragment amplification and sequencing. We used fragments of the following four genes for phylogenetic analysis: the mitochondrial cytochrome oxidase subunit I (COI) gene, 16S mitochondrial rRNA gene, FASTKD4 nuclear gene, and the 28S nuclear rRNA gene. Sanger sequencing was performed by Microsynth France SAS (Vaulx-en-Velin, France). New PCR primers specifically designed as part of this study for sequencing the FASTKD4 nuclear gene are provided in Suppl. material 1: table S1. Accession numbers of the 118 DNA sequences generated as part of this study are provided in Suppl. material 1: table S10. Throughout the text, we refer to species (either formally described or awaiting formal description) identified based on morphological criteria as morphospecies, and to putative species identified based on molecular criteria as molecular operational units (MOTUs).

Molecular species delimitation and within-species divergence

MOTUs were delimited based on a COI alignment of 137 haplotypes, which were defined from the sequences obtained from 165 specimens belonging to 26 morphospecies of Stenasellidae collected at 97 sampling sites located in Africa (9 sites, 5 morphospecies), America (2, 2), Asia (12, 7) and Europe (74, 12) (Suppl. material 1: tables S2, S3). The longest sequence with the fewest ambiguities was retained as the best representative sequence for any given haplotype.

Six different species delimitation methods were used to delimit MOTUs: the Automatic Barcode Gap Discovery (ABGB, Puillandre et al. 2012), the Assembling Species by Automatic Partitioning method (ASAP, Puillandre et al. 2021), the Poisson Tree Processes method (PTP) and its Bayesian (bPTP; Zhang et al. 2013) and multi-rate (mPTP; Kapli et al. 2017) implementations, and the fixed COI threshold method (TH, implemented by Lefébure et al. (2006) for crustaceans). ABGD and ASAP methods were run on the Spart Explorer server (https://spartexplorer.mnhn.fr) using the COI haplotype alignment and all default parameters but 1 as a relative gap width

(ABGD: X = 1, Pmin =0.001, Pmax=0.1 and 10 steps; ASAP: Split threshold = 0.001). We applied the PTP and TH methods to a COI haplotype phylogeny constructed in maximum likelihood with IQ-TREE 3 (Wong et al. 2025). The best model of evolution was selected with ModelFinder (Kalyaanamoorthy et al. 2017). MOTUs were delimited with the PTP and bPTP methods in the dedicated server (https://species.h-its.org/ptp) using 400,000 Markov chain Monte Carlo (MCMC) generations, with a thinning of 400 and 0.1 (10%) burn-in. We ran mPTP v0.2.2 on the dedicated server (https://mptp.h-its.org/#/tree) to delimit MOTUs using the mPTP method. Patristic distances from the COI haplotype phylogeny were computed with the R package "ape" (Paradis et al. 2004) and MOTUs were delimited according to the TH method with the "cluster" package (Maechler et al. 2025). Spart Explorer server was used to compare graphically the sets of MOTUs obtained with the six molecular species delimitation methods. For the sake of continuity, MOTU were numbered following Saclier et al. (2024). To estimate evolutionary divergence among and within populations (sampling sites) of Stenasellidae collected in Cambodia and Vietnam, we computed the uncorrected genetic distances (*p*-distance) for the COI and 28S genes and patristic distances for the COI gene for all available sequence pairs.

Four-gene phylogeny of the Stenasellidae

We constructed a four-gene phylogeny of the Stenasellidae using MOTUs delimited with the TH method to enable comparison with previously published phylogenies of the Stenasellidae (Morvan et al. 2013; Jugovic et al. 2024; Saclier et al. 2024). Alignments of the COI, 16S, FASTKD4 and 28S gene markers were generated for 48 MOTUs of Stenasellidae (Suppl. material 1: tables S4–S8). MOTUs of Stenasellidae in the phylogeny were from Africa (5 MOTUs; genera *Magniezia* and *Metastenasellus*), Asia (7 MOTUs; genus *Stenasellus*), and Europe (36 MOTUs; genera *Balkanostenasellus*, *Protelsonia* and *Stenasellus*). Five MOTUs belonging to distinct morphospecies of Asellidae were used as outgroup, namely *Asellus* (*Asellus*) *aquaticus* Linnaeus, 1758, *Bragasellus lagari* Henry & Magniez, 1973, *Caecidotea stygia* Packard, 1871, *Proasellus cavaticus* (Leydig, 1871), and *Salmasellus howarthi* Lewis, 2001 (Suppl. material 1: table S8). For the four gene alignments, we used the chimera assembler script (https://github.com/TristanLefebure/chimera_assembler.pl) for selecting the longest sequence with the fewest ambiguities to represent each MOTU. Sequences of the 28S and 16S genes were aligned with MAFFT Q-INS-i (Katoh and Standley 2013) and the COI and FASTKD4 genes were aligned with Clustal Omega (Sievers et al. 2011). Sites ambiguously aligned were selected and removed using Gblocks (Castresana 2000) and final adjustments were made by hand. Four genes for 53 MOTUs were used to build phylogenies with both PhyloBayes (Lartillot et al. 2009) and IQ-tree3 (Wong et al. 2025). PhyloBayes was ran under a CAT-GTR model of evolution using two independent chains; convergence between chains was checked with the bpcomp program and consensus tree was built using one tree every 10 generations, a burn-in of 1000 and a maxdiff threshold of 0.1. A cutoff rule to 0.1 was set to avoid polytomies and

posterior probabilities (pp) were used to estimate the support for tree topologies. IQ-tree3 was ran using eight partitions (i.e. one partition per gene and codon position for protein coding genes) and the best models of evolution as identified by ModelFinder (Kalyaanamoorthy et al. 2017). Five hundred bootstrapping were computed to estimate the support of tree topologies. Considering that the COI third position showed signs of saturation (e.g. IQ-tree estimated that this partition evolved more than 60 times faster than the second fastest partition), we produced four-gene phylogenies with and without that position in PhyloBayes (Lartillot et al. 2009) and IQ-tree3.

Species description

We described the habitus, alive coloration, detailed morphology, and provided scanning electron microscopy (SEM) photographs of *Stenasellus angiangensis* sp. nov. collected in southern Vietnam. For the habitus and alive coloration, freshly collected specimens were relaxed in a clove oil and then photographed using a Canon G16 digital camera. For detailed morphology, fixed specimens were dissected under a Lomo MBS-10 stereomicroscope. The body parts were then mounted on slides in glycerin or polyvinyl lactophenol, covered with a cover slip and sealed with transparent nail polish. Photos of body parts were taken using a digital Olympus ZX10 camera connected to an Olympus CX21 compound microscope. Any body parts that had been blurred due to the high depth of field were sharpened and specimen borders of body parts were outlined in Photoshop, whenever necessary. Enhanced photos have an advantage for species description over traditional drawings in that they minimize interpretations that could distort reality. SEM images were obtained using the Vega3 Tescan microscope at the Paleontological Museum of the Paleontological Institute of the Russian Academy of Sciences, Moscow. Specimens were placed in 95% ethanol, cleaned in an ultrasonic cleaner, followed by dehydration with acetone and critical-point drying (CPD). Subsequently, they were affixed to specimen stubs with double-sided tape, and finally coated with gold through sputtering using the Polaron PS 100.

Body length (bl., is mm) was measured from the anterior margin of the cephalothorax to the posterior margin of the pleotelson; body width (bw, in mm) was measured as the maximum distance between lateral margins of any segment of the pereon.

Results

Phylogeny and molecular taxonomy

Phylogenetic analysis of the family Stenasellidae revealed six strongly supported polytypic clades (bootstraps >90%; posterior probabilities >0.9) and two monotypic clades corresponding to *S. asiaticus* and *Magniezia gardei* Magniez, 1978 (Figs 1B, 2). Among them, Clade 2 contained all Asian MOTUs, except *S. asiaticus*. *S. angiangensis* sp. nov. was recovered as the sister species to a lineage comprising the two geographically

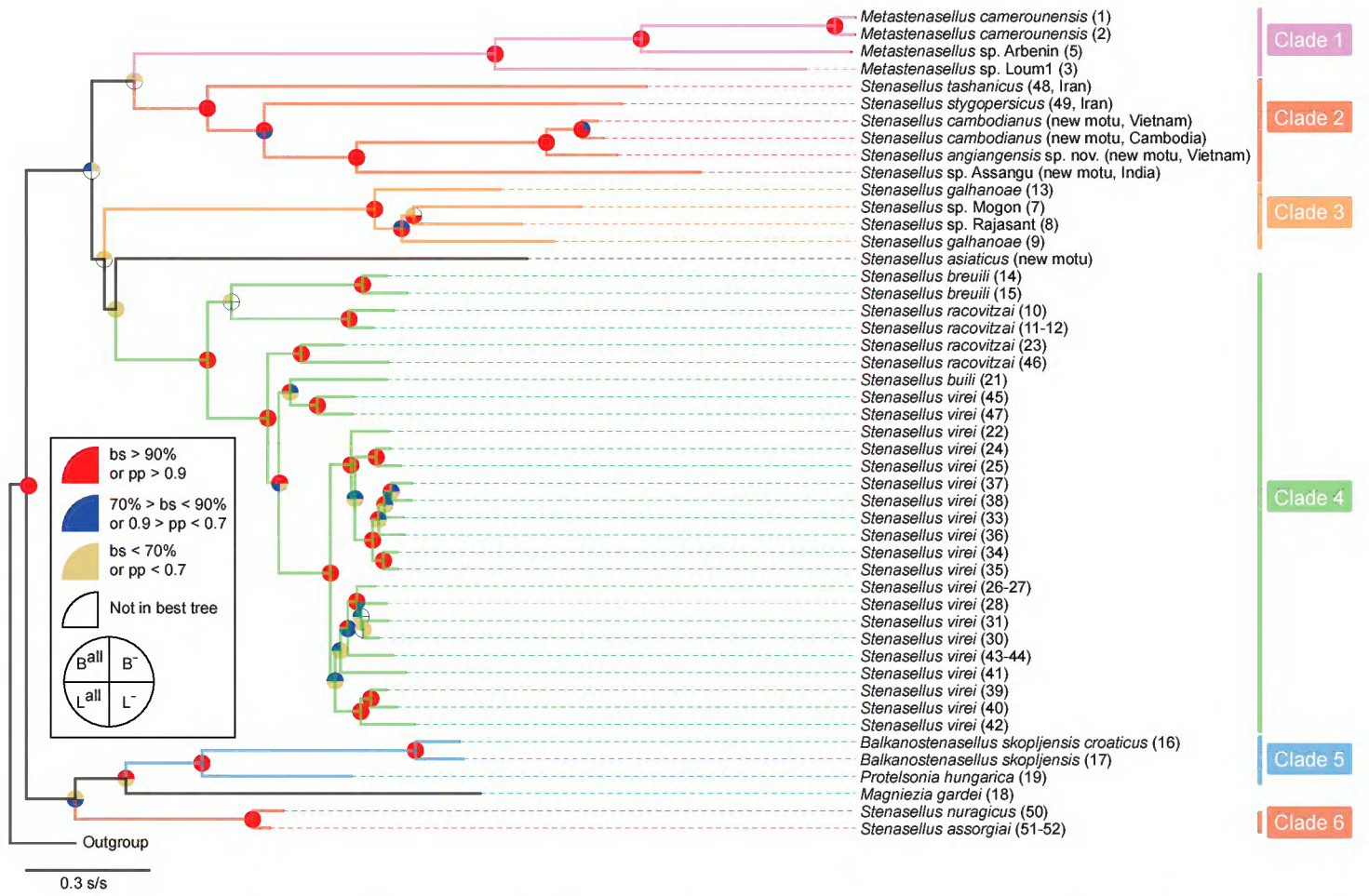

Figure 2. Four-gene phylogeny of Stenasellidae (Isopoda, Pancrustacea) collected in Africa, Asia and Europe. The tree is rooted using five species of Asellidae as outgroup (species not shown, see text). Terminal nodes are MOTUs as delimited with the fixed COI threshold method. Pie charts show node supports obtained with IQ-TREE 3 (L: Maximum-Likelihood phylogenetic inferences; bs: bootstraps) and PhyloBayes (B: Bayesian phylogenetic inferences; pp: posterior probabilities). Node supports are provided for phylogenetic inferences performed with (all) and without (-) the COI third position. Well-supported clades within Stenasellidae are indicated on the right margin.

separated MOTUs of *S. cambodianus*. Clade 1 was composed of MOTUs belonging to the African genus *Metastenasellus*. Clade 3 grouped four MOTUs of *Stenasellus* from southern Spain and southern Portugal. Clade 4 comprised 27 MOTUs of *Stenasellus* from southern France (including Corsica), northern Spain, and Italy (including northern Sardinia). Clade 5 grouped MOTUs belonging to the genera *Balkanostenasellus* (Slovenia and Croatia) and *Protelsonia* (Hungary). Clade 6 contained two MOTUs corresponding to morphological species described from Sardinia, *Stenasellus nuragicus* Argano, 1968 and *Stenasellus assorgiai* Argano, 1968. The phylogenetic relationships among the eight clades were not resolved, suggesting that deeper divergences within the family Stenasellidae remain uncertain.

Among the six molecular species delimitation methods applied, the mPTP yielded the lowest number of putative species within Stenasellidae, namely 44 MOTUs. This number was lower than those produced by TH (53 MOTUs) ASAP (66), ABGD (69), PTP (74) and bPTP (77) methods (Fig. 3). All methods consistently assigned *S. angiangensis* sp. nov. and *S. cambodianus* to distinct MOTUs (Fig. 4). The mPTP and TH methods grouped all specimens of *S. angiangensis* sp. nov. from its three known sites into a single MOTU (Fig. 4). In contrast, the other four methods consistently assigned

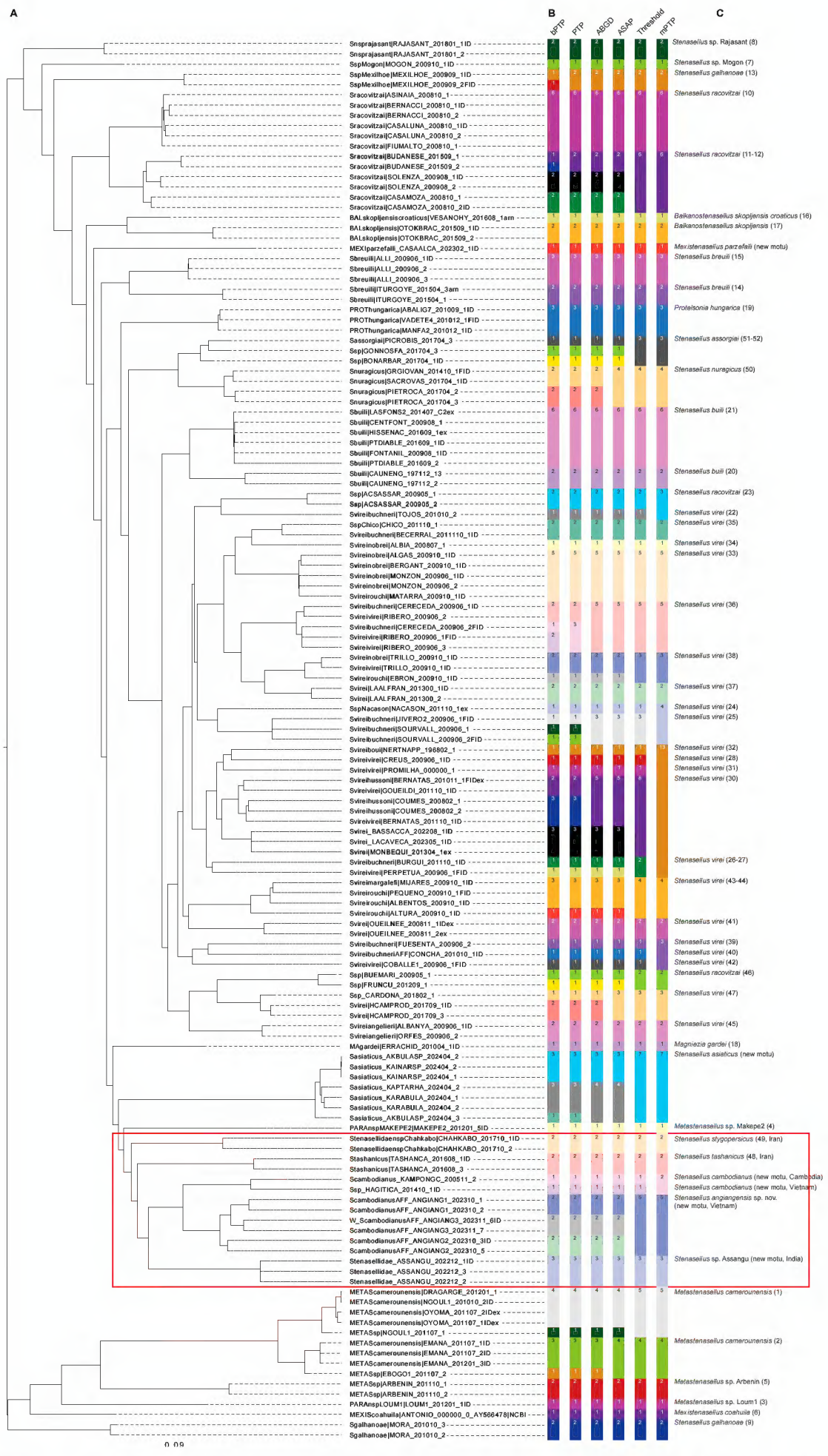

Figure 3. Delimitation of molecular operational taxonomic units (MOTUs) of Stenasellidae. **A** phylogeny using the COI gene fragment **B** results of COI-based molecular species delimitation using the Poisson Tree Processes method (PTP) and its Bayesian implementation (bPTP), the Automatic Barcode Gap Discovery method (ABGD), the Assembling Species by Automatic Partitioning method (ASAP), the fixed threshold method (TH), and the multi-rate PTP (mPTP) **C** taxon names corresponding to MOTUs as delimited with TH. Numbers in parentheses refer to MOTU numbers as defined by Saclier et al. (2024). The red box shows Asian stenasellidae (see Fig. 4), except *S. asiaticus*.

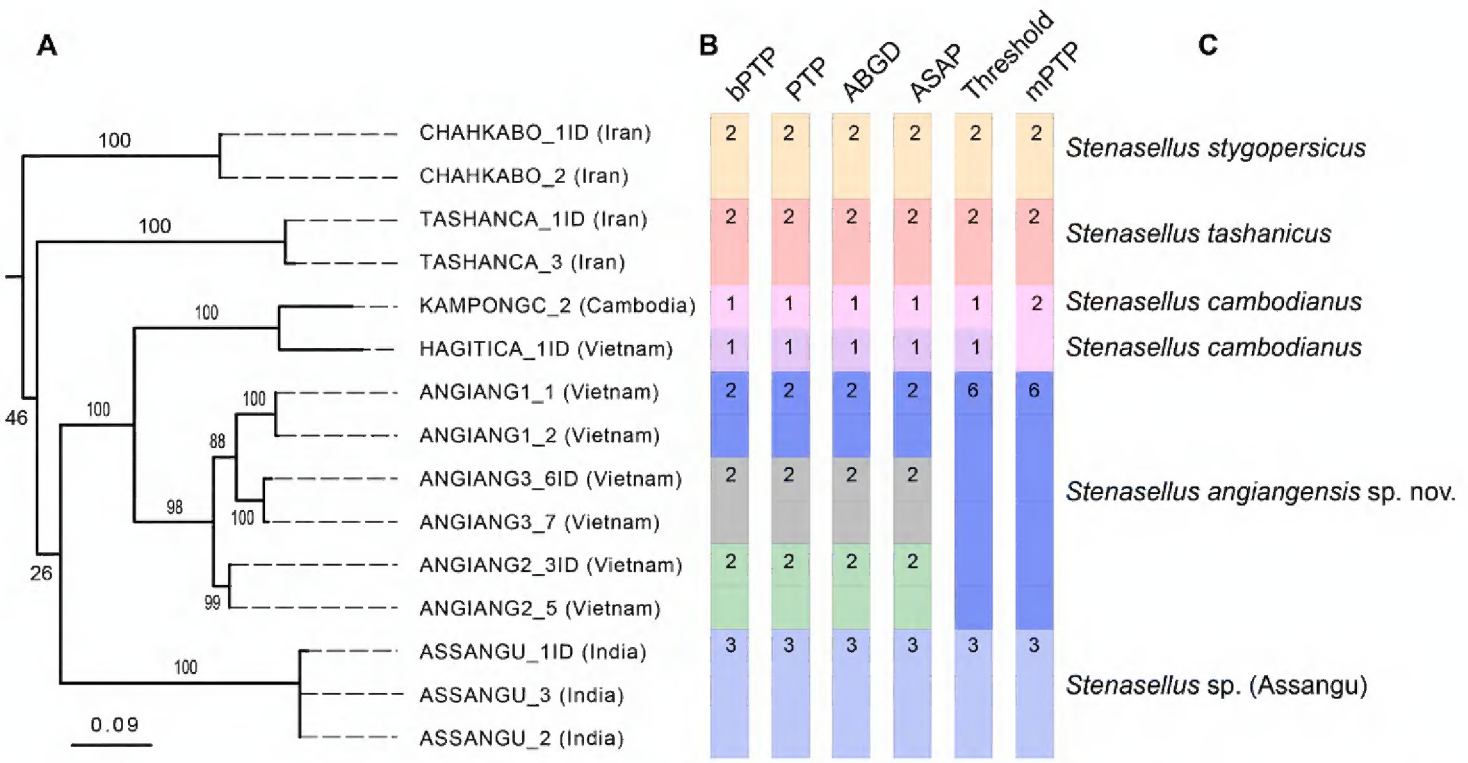

Figure 4. Delimitation of molecular operational taxonomic units (MOTUs) of Stenasellidae in Asia (except *S. asiaticus*, see Fig. 3 for a delimitation of MOTUs of the entire Stenasellidae). **A** phylogeny using the COI gene fragment **B** results of COI-based molecular species delimitation using the Poisson Tree Processes method (PTP) and its Bayesian implementation (bPTP), the Automatic Barcode Gap Discovery method (ABGD), the Assembling Species by Automatic Partitioning method (ASAP), the fixed threshold method (TH), and the multi-rate PTP (mPTP) **C** taxon names corresponding to MOTUs as delimited with TH. Branch supports are bootstrap approximations based on 1000 replicates.

each site of this new species to a distinct MOTU. All species delimitation methods, except mPTP, consistently assigned the populations of *S. cambodianus* from Cambodia (site code: KAMPONGC) and Vietnam (HAGITICA) into two distinct MOTUs (Fig. 4). mPTP combined these two populations into a single MOTU.

The three populations of *S. angiangensis* sp. nov. exhibited relatively high pairwise genetic divergences (*p*-distance = 0.059±0.003, patristic distance = 0.08±0.004), despite the relatively short geographic distances among the three sites (maximum distance = 10.2 km) (Suppl. material 1: table S9). Genetic distances between sites located on the different sides of the same hill (ANGIANG 2 and 3, separated by 2.6 km) were comparable to those between these two sites and the more distant site ANGIANG 1 (7.9 and 10.2 km), which was located on a separate hill (Suppl. material 1: table S9). The genetic distance measured for the COI gene between the two populations of *S. cambodianus*, geographically separated by approximately 53 km, was 0.12 (patristic distance: 0.18) (Suppl. material 1: table S9).

Pairwise genetic distances for the 28S gene between populations of *S. angiangensis* sp. nov. and *S. cambodianus* (p=0.04105 ±0.00277, n=4) were 5 times greater than between populations of *S. cambodianus* (p=0.0080) (Suppl. material 1: table S9). We found no divergence (p=0) between populations of *S. angianensis* sp. nov. at ANGIANG 2 and ANGIANG 3 for the 28S gene.

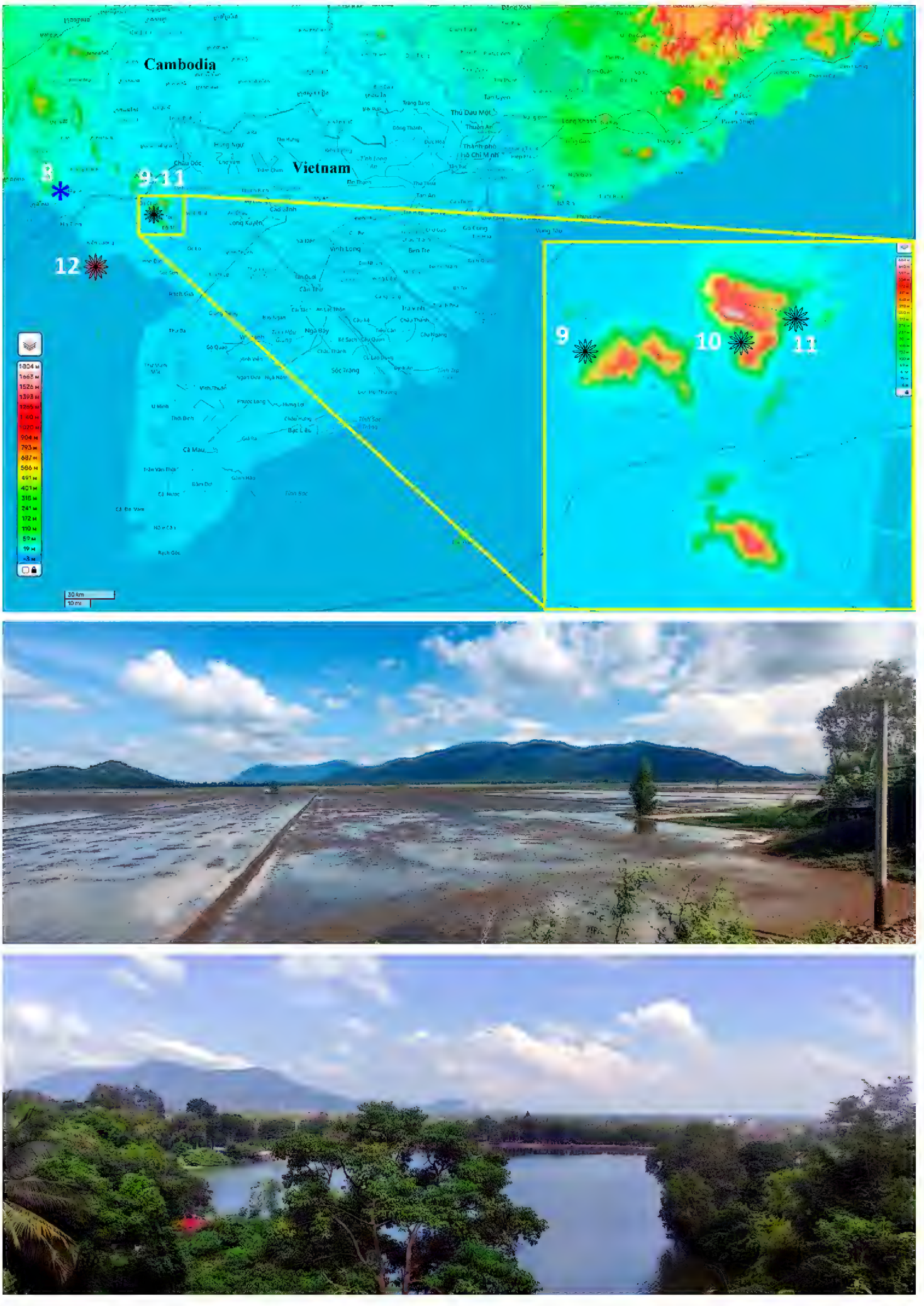

Figure 5. Upper panel: Map showing the location of sites 9–11 where *Stenasellus angiangensis* sp. nov. was found. Numbers 8 and 12 show location of sites where *Stenasellus cambodianus* was collected. Lower panels: general view of isolated mountains in the Mekong delta, An Giang Province, south west of Vietnam.

Species description

Order Isopoda Latreille, 1816
Suborder Asellota Latreille, 1802
Family Stenasellidae Dudich, 1924
Genus *Stenasellus* Dollfus, 1897

***Stenasellus angiangensis* Marin, Palatov, Thinh, Douady & Malard, sp. nov.**
https://zoobank.org/997DB87C-7A52-4B3C-932F-BDCD2760A306
Figs 6b–d, 11

Material examined. ***Holotype*:** • ♂, bl. 5.1 mm, ZMMU Mc-1471 – **Vietnam**, An Giang Province, Tri Tôn, 10°29'05.7"N, 104°54'46.9"E, in hyporheic habitats of a small mountainous river, located on a mountainside overgrown with forest (site number and code: 9, ANGIANG1), hand net sampling, coll. D. Palatov & I. Marin, 10 November 2023; Paratype, ♀, bl. 4.7 mm, ZMMU Mc-1472 – same locality and date as for holotype.

***Paratypes*:** • 1♂, 1♀ (ZMMU Mc-1473), 1♂, 1♀ (LEMMI, used for SEM), 1♂, 2♀♀ (including 1 posterior part of a female) (UCBLZ: Coll Number 2012.11.25.88) – Vietnam, An Giang Province, Tri Tôn, 10°29'47.8"N, 105°00'20.1"E, in hyporheic habitats of a small mountainous river, located on a mountainside overgrown with forest (11, ANGIANG2), hand net sampling, coll. D. Palatov & I. Marin, 10 November 2023.

Additional material. Vietnam • 3♀♀, 2♂♂ (LEMMI), 1♀, 1 head of specimen (UCBLZ: Coll Number 2012.11.25.87) – An Giang Province, Tri Tôn, 10°29'05.7"N, 104°54'46.9"E, in hyporheic habitats of a small mountainous river, located on a mountainside overgrown with forest (9, ANGIANG1), hand net sampling, coll. D. Palatov & I. Marin, 11 September 2023; • 2♀♀ (LEMMI), 1♀, 2♂♂(UCBLZ: Coll Number 2012.11.25.89) – 10°29'00.1"N, 104°59'09.6"E, in hyporheic habitats of a small mountainous river, located on a mountainside overgrown with forest (10, ANGIANG3), hand net sampling, coll. D. Palatov & I. Marin, 11 November 2023.

Etymology. The species name, *angiangensis*, is given after An Giang Province of southern Vietnam, where the species was discovered.

Diagnosis. Body dorsal surface smooth, with small scattered marginal setae. Antennula and antenna relatively short, with 8 and 26 flagellar articles, respectively. Disto-mesial margin of endite of maxilliped with 2 coupling hooks. Male pleopod I with protopod without coupling hook on inner margin. Distal end of exopod of male pleopod I bluntly convex. Male pleopod II with almost quadrate protopod with convex external margin; exopod with small and subquadrate proximal article with one small seta on external margin; distal article of exopod oval with 6 simple setae along external margin. Endopod of male pleopod II with distal article subequal to basal article, without spines or setae; terminal end of the distal article of endopod bearing a flute-shaped

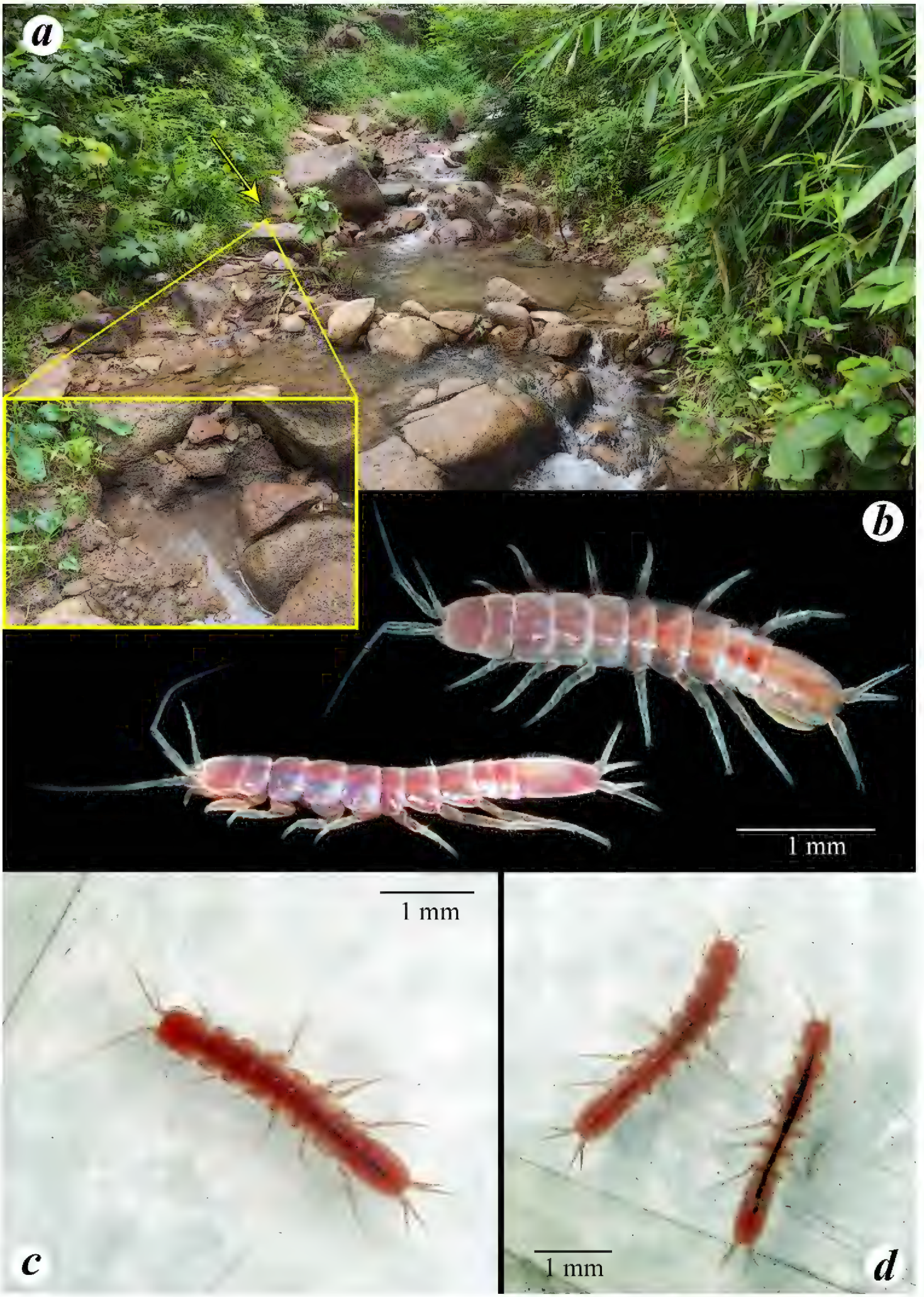

Figure 6. The habitat (**a**) and general view (**b–d**) of *Stenasellus angiangensis* sp. nov. in the An Giang Province, Mekong Delta area in southern Vietnam. Specimens were collected using a hand net in the hyporheic zone and springs of mountainous streams.

efferent canal and a strong spur, the terminal end of which reaches the terminal end of the efferent canal. Endopod of pleopods III, IV, and V deeply bifurcated.

Description. Holotype ♂ (ZMMU Mc-1471). Body elongated, about 6.0× longer than wide (Fig. 6b). Head trapezoidal, about 1.2× wider than long, with straight frontal margin, dorsal surface smooth, with small marginal setae. Pereonite I about 2.2× wider than long, shorter that pereonites II–IV; pereonites II–IV subequal in length, about 2.3–2.5× wider than long, with rounded lateral margins; pereonites V–VII subequal in length, about 1.5–2.2× wider than long, slightly longer than pereonites II–IV, with posterolateral margins projected posteriorly. Pleonites I–II (Fig. 10a) subequal in length, about 2.8–3.0× wider than long, with projected posterolateral corner, furnished with strong blunt spines and simple setae.

Coloration. Characteristic for other known congeneric species; deeply pink or red (see Fig. 6b–d).

Body size. The average body length differed significantly between males and females: males averaged 4.43±0.21 mm (min: 4.23; max: 4.80; n=7), while females averaged 4.87±0.46 mm (min: 4.15; max: 6.00; n=7). Similarly, the average body width also showed a significant difference, with males measuring 0.81±0.01 mm (min: 0.80; max: 0.82; n=6) and females 0.88±0.07 mm (min: 0.80; max: 1.10; n=12). In both cases, the differences were statistically significant ($p < 0.05$). These results indicate that females are significantly larger than males in terms of both body length and body width. Body is 5.4±0.2 × longer than wide (n=18 specimens), with no difference between males and females.

Antennula (Fig. 7a, b) relatively short, reaching pereonite II, slightly longer than the peduncle of antenna; peduncle 4-articulated, article I stout, about 1.2× longer than wide, with 4 strong setae on distal margin, article II about 1.8× longer than wide, with strong simple and several plumose setae, article III about 1.7× longer than wide, with 2 distal glabrous and 1 long plumose setae, article IV short, about as long as wide, with 1 plumose setae; flagellum with 8 articles, five distal articles bearing a single aesthetasc.

Antenna (Fig. 7c) with 6-articulated peduncles; articles I–II reduced, article II about 2.0× wider than long; articles III–V relatively stout, slightly increasing in length, with length proportion articles III–VI close to 1.0/0.8/1.2/1.8; article III about 1.5× longer than wide, armed with long simple setae distally; article IV about 1.6× longer than wide, with minute squama bearing stout setae, article V about 2.3× longer than wide, armed with several stout and numerous simple setae, article VI about 4.2× longer than wide, covered with simple setae; flagellum about 1.8× longer than peduncle, reaching to pereonite IV, with 26 articles.

Labrum (upper lip) (Fig. 7d) oval, apical margin convex, with numerous small fine setae.

Labium (lower lip) (Fig. 7e) with feebly developed inner lobes.

Mandibles. Right mandible (Fig. 8a, b) with incisor process bearing 4 teeth and sclerotized lama, *lacinia mobilis* strong and stout, with 5 blunt teeth; molar process with 12 long setae. Left mandible (Fig. 8c, d) with incisor process bearing 3 teeth and sclerotized lama, *lacinia mobilis* feebly developed; molar process with 12 long setae. Mandibular palp (Fig. 8a, c) 3-articulated, basal article I with 1 long seta on distal

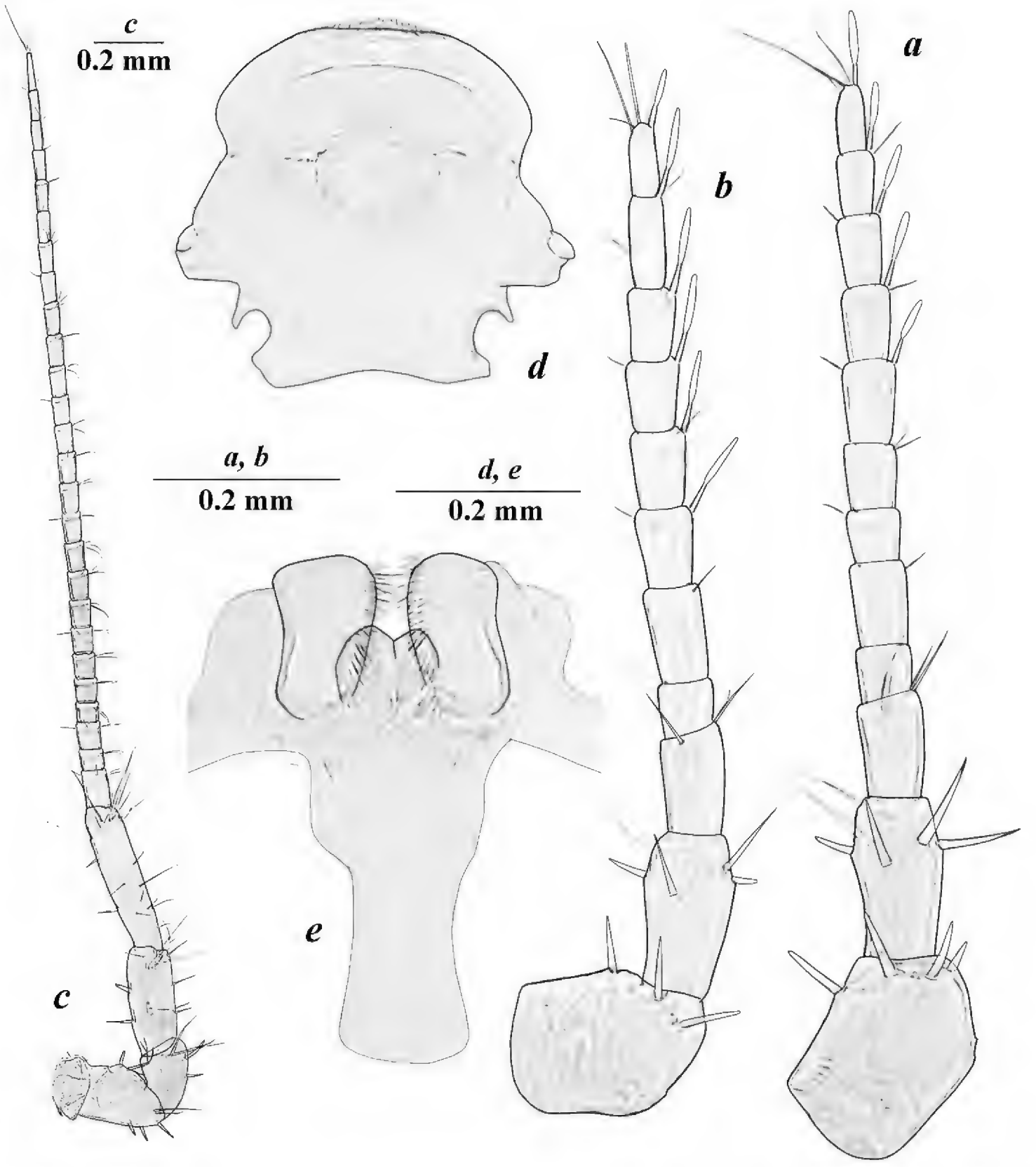

Figure 7. *Stenasellus angiangensis* sp. nov., paratype ♀ (ZMMU Mb-1472) (**a, d, f–h**) and holotype ♂ (ZMMU Mc-1471) (**b, c, e**). **a, b** antennula **c** antenna **d** labrum **e** labium.

corner, article II with 7–8 setae on internal margin, and terminal article III with almost 10 setae situated in two rows and 2 long distal simple setae.

Maxillula (Fig. 8e) with 13 distally curved pectinate robust setae in two rows on outer lobe (Fig. 8f); with 2 strong scarcely plumose robust combs and 1–2 curved pectinate simple setae on inner lobe.

Maxilla (Fig. 8g) lateral and middle endites each with 8 curved pectinate robust setae; mesial endite with 11 long robust plumose combs and several slender simple setae.

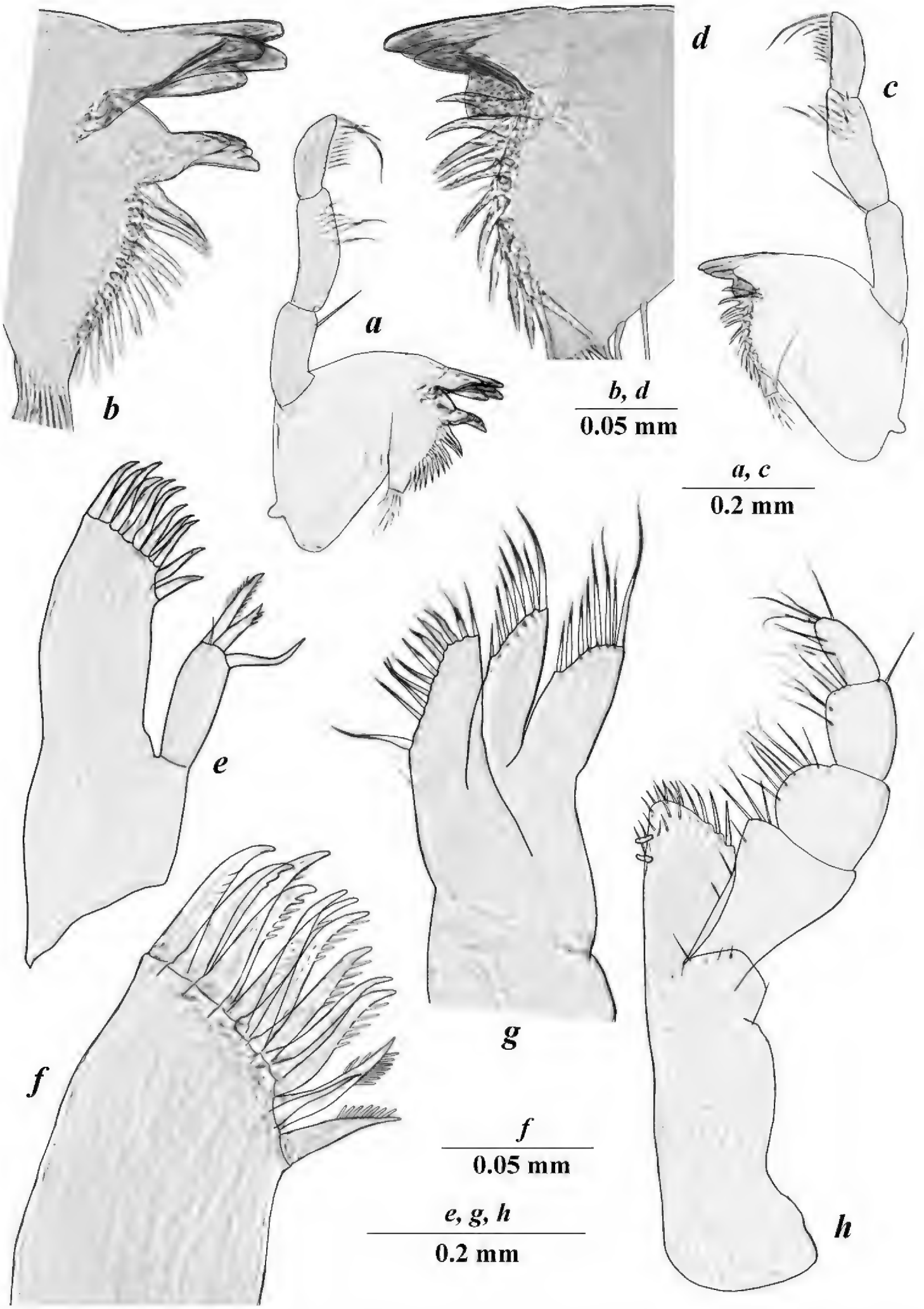

Figure 8. *Stenasellus angiangensis* sp. nov., holotype ♂ (ZMMU Mc-1471). **a** right mandible **b** same, incisor process **c** left mandible **d** same, incisor process **e** maxillula **f** outer lobe of maxillula **g** maxilla **h** maxilliped.

Maxilliped (Fig. 8h) with disto-mesial margin of endite bearing 2 coupling hooks and numerous robust setae of different length; palp 4-articulated, with articles armed with numerous long simple setae along inferior margins.

Pereopod I (Fig. 9a) haptorial, with robust dactylar ventral claw; coxa about as long as wide, with 3 long marginal setae; basis about 2.0× longer than wide, with inner margin distinctly concave proximally; ischium about 1.4× longer than wide, with 1 strong medial seta on external margin; merus with supradistal angle bearing 1 strong seta, inner margin distally with 2 short robust and several simple setae; carpus triangular, inferior margin covered with numerous long and short robust setae and several simple setae; propodus about 2.1× as long as wide, somewhat teardrop-shaped, inferior margin covered with several robust and serrated setae; dactylus 4.8× as long as basal width, inferior margin with row of contiguous scale-like flattened setae with accessory setulae, main unguis elongate, distally sharpening.

Pereopod II (Fig. 9c) with coxa about as long as wide, with tiny marginal setae; basis about 2.0× longer than maximal width, swollen in central part, with external margin distinctly convex, armed with 4 strong setae proximally, inner margin with small median setae; ischium about 1.7× longer than wide, with external margin armed with 3 long robust medial and 3 subdistal short setae; merus about as long as wide, subtriangular in shape, with supradistal angle bearing 3 strong setae, inner margin distally with 2 short robust and several simple setae; carpus subcylindrical, about 2.5× longer than wide, widening proximally, inferior margin covered with numerous long and short robust setae and several simple setae; propodus about 4.8× as long as wide, inferior margin covered with several robust and serrated setae; dactylus shorter than main unguis, with single long accessory spine on sternal margin.

Pereopods III–VII (Fig. 9d–h) generally similar in shape with pereopod II; pereopod length progressively increasing from pereopods II to VII; coxa about as long as wide, with tiny marginal setae; basis about 2.2× longer than maximal width in PIII–PIV and about 1.6×-1.7× in PV–PVII, swollen in central part, with external margin distinctly convex, armed with 3 strong setae proximally, inner margin with small median setae; ischium about 1.6× in PIII and about 1.9×-2.1× in PIV–PVII, longer than wide, external margin armed with 3 long robust medial and 3 subdistal short setae in PIII and PIV, with 5 long robust medial setae in PV–PVII; merus about as long as wide, subtriangular in shape, with supradistal angle bearing 3 strong long setae and inner margin distally with 3 short robust and several simple setae in PIII–PIV and supradistal angle with 5 strong long setae, inner margin distally with 3–4 short robust and several simple setae in PV–PVII; carpus subcylindrical, about 2.7×–3.0× longer than wide, widening proximally, inferior margin covered with numerous long and short robust setae and several simple setae in PIII–PV and inferior margin covered with 2 long median and 2 subdistal robust setae accompanying with several simple setae in PVI and PVII; propodus about 4.8×–5.2× as long as wide, inferior margin covered with several robust and serrated setae; dactylus shorter than main unguis, with single long accessory spine on sternal margin.

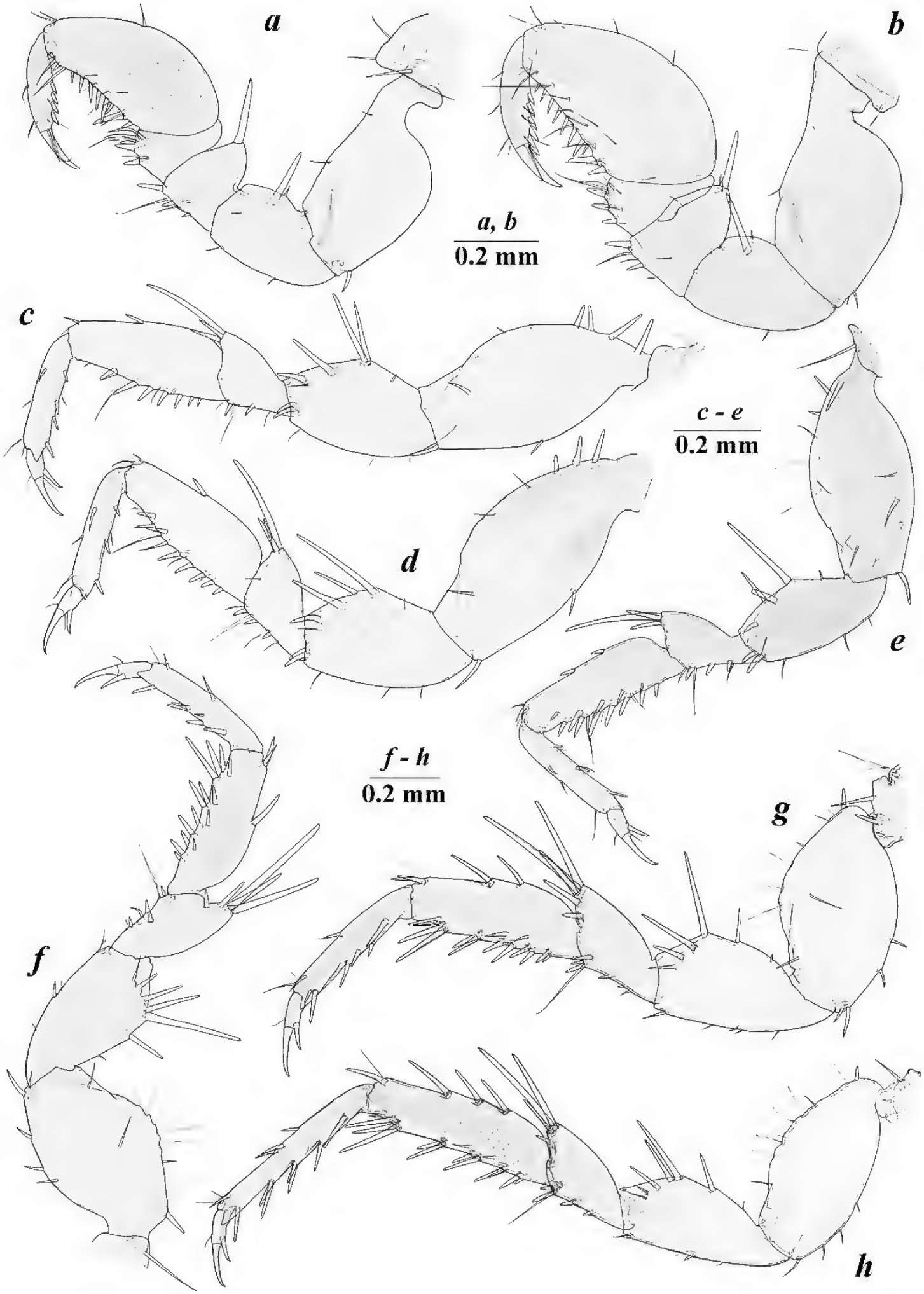

Figure 9. *Stenasellus angiangensis* sp. nov., holotype ♂ (ZMMU Mc-1471) (**a, c–h**) and paratype ♀ (ZMMU Mb-1472) (**b**). **a, b** pereopod I **c** pereopod II **d** pereopod III **e** pereopod IV **f** pereopod V **g** pereopod VI **h** pereopod VII.

Pleotelson elongated, about 1.5× longer than broad, with scattered marginal setae; posterior margin with two slight excavations extended in the center, bluntly rounded (Fig. 10b).

Pleopod I (Fig. 10d) with protopod about as long as wide, without coupling hook on inner margin; exopod elongated, bluntly rounded distally, shovel-like, mesial margin with a row of 12 simple setae, apical margin with several tiny setae, lateral margin convex.

Pleopod II (Fig. 10e) almost quadrate protopod about as long as wide, external margin convex, with one paramedial spine; exopod with small and subquadrate proximal article, with small seta on external margin, distal article oval, about as long as wide, with 6 simple setae along external margin; endopod (Fig. 10f) with slender articles, distal article subequal to basal article, without spines or setae. Terminal end of the distal article of endodpod bearing a flute-shaped efferent canal and a strong spur, the terminal end of which reaches the terminal end of the efferent canal.

Pleopod III (Fig. 10g) with endopod 0.8× as long as exopod, deeply bifurcated distally; exopod about 2.5× as long as wide, with distinct transverse suture, apical margin bluntly convex, with several slender simple setae. Pleopod IV (Fig. 10h) with endopod slightly longer than exopod, deeply bifurcated distally; exopod about 2.2× as long as wide, with transverse suture, distolateral margin with 17 slender simple setae. Pleopod V (Fig. 10i) similar to pleopods III–IV, endopod subequal in length to exopod, deeply bifurcated distally; exopod about 2.4× as long as wide, with transverse suture, distolateral margin with numerous tiny setae.

Uropods (Fig. 10j) as long as pleotelson, protopod about 1.5× longer than wide, covered with strong spines; endopod longer than exopod, endopod about 9.0× as long as wide, covered with long distal glabrous setae; exopod about 7.7× as long as wide, covered with long distal glabrous setae.

Penial processes (Fig. 10k) elongated, cylindrical, sharping distally, about 3.8× longer than greatest width.

Females. Pleotelson and antenna are subequal to those in ♂♂. Pereopod I (Fig. 9b) haptorial, with ventral claw, generally similar but with articles slightly slender than in ♂♂. Pleopod II (Fig. 10c) with protopod triangular in shape, about as long as wide, with several simple setae along inner margin.

Taxonomic remarks. Morphologically, the new species is most closely similar to *Stenasellus cambodianus* Boutin & Magniez, 1985, which records are also most close geographically, being known from a cave of the Kampong Trach karst in southeastern Cambodia (see Boutin and Magniez 1985) and the cave Hang Giếng Tiên of the Hòn Chông karst in Kiên Giang Province of southern Vietnam (Deharveng et al. 2023). Both species share a strong spur on the segment of endopod of pleopod II in males, which makes it possible to separate them from other representatives of the genus *Stenasellus*. However, *Stenasellus angiangensis* sp. nov. is easily distinguishable from *S. cambodianus* and other species of *Stenasellus* due to the flute-shaped terminal end of the efferent canal (copulatory organ, see Fig. 10f). As most species in Asia (Magniez

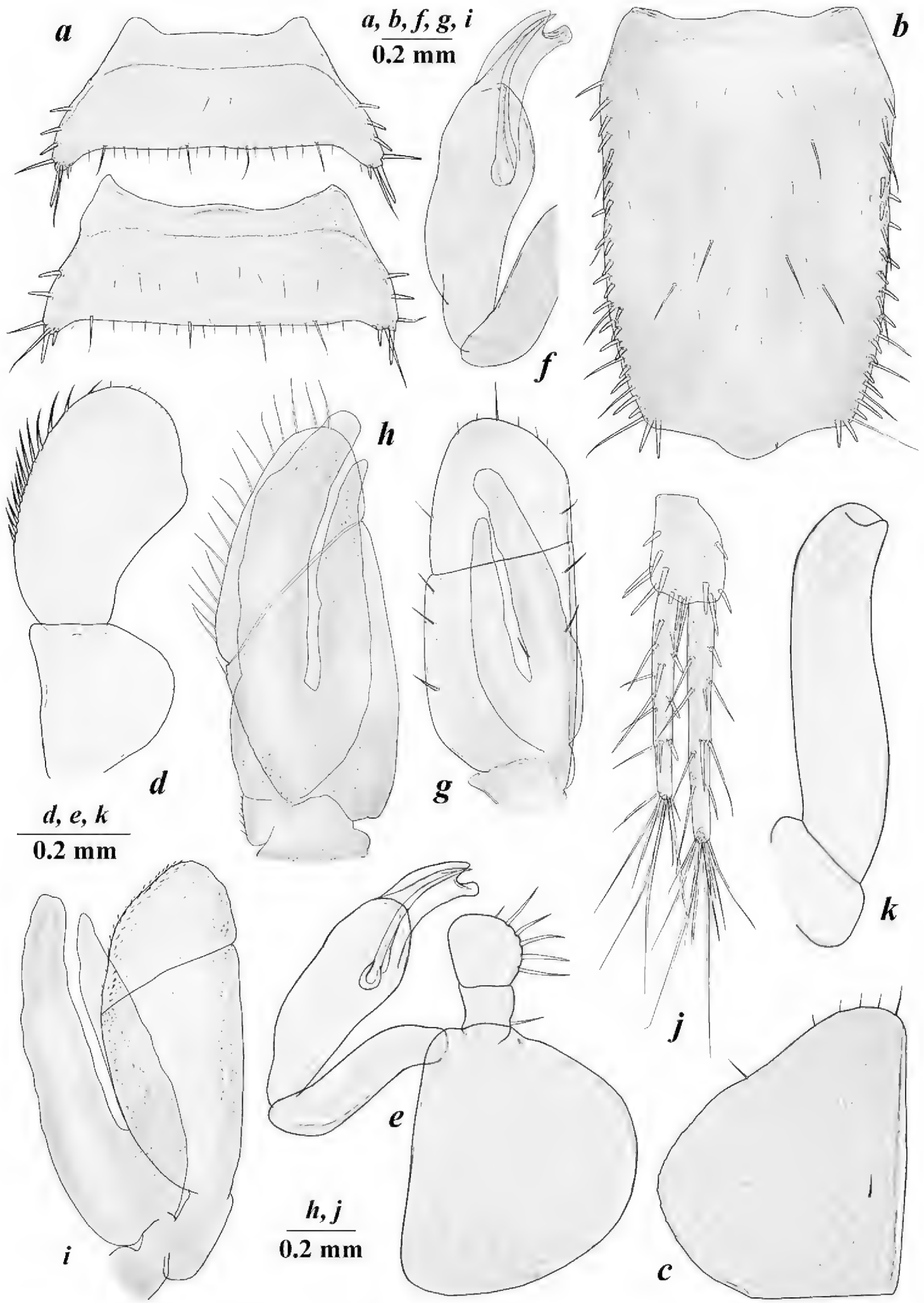

Figure 10. *Stenasellus angiangensis* sp. nov., holotype ♂ (ZMMU Mc-1471) (**a, b, d–k**) and paratype ♀ (ZMMU Mb-1472) (**c**). **a** pleonites I and II **b** pleotelson, dorsal view **c** pleopod II **d** pleopod I **e, f** pleopod II **g** pleopod III **h** pleopod IV **i** pleopod V **j** right uropod, dorsal **k** penis.

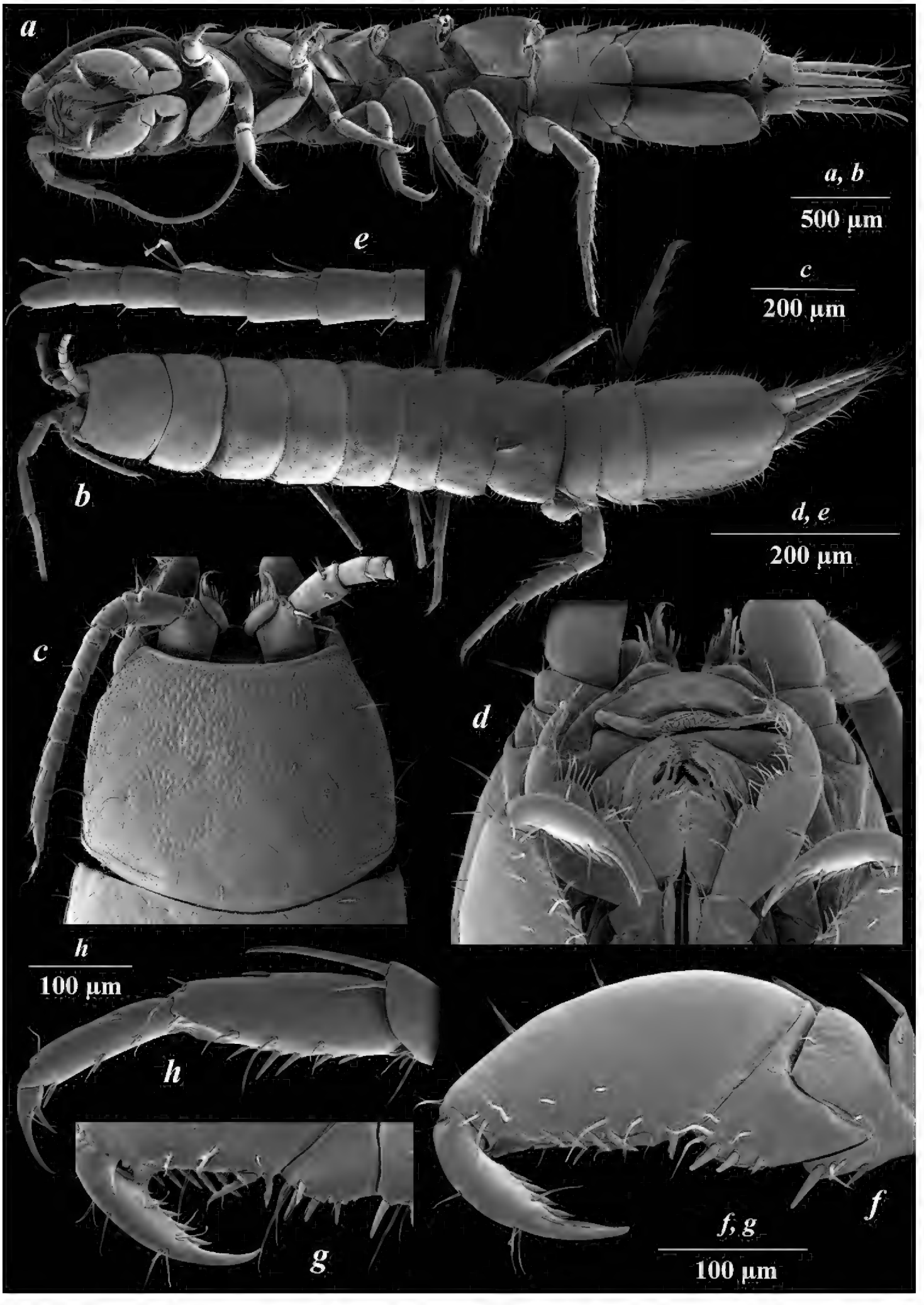

Figure 11. *Stenasellus angiangensis* sp. nov., ♀ (**a–e, h**) and ♂ (**f, g**), LEMMI, SEM photographs. **a** ventral general body view **b** dorsal general body view **c** head **d** oral appendages **e** antennular flagellum **f** distal segments of pereopod I **g** spinulation of distal segments of pereopod I **h** distal segments of pereopod II.

2005), endopods of pleopods III, IV and V are bifurcated in the new species, even deeply than in *S. cambodianus* (see Boutin and Magniez 1985). Indeed, all *Stenasellus* from Asia have bifurcated endopods III, IV and V except *Stenasellus asiaticus* Birstein & Starostin, 1949 (Turkmenistan), *Stenasellus boutini* Magniez, 1991 (Cambodia), and three species from Thailand (*Stenasellus deharvengi* Magniez, 1991, *Stenasellus rigali* Magniez, 1991 and *Stenasellus mongnatei* Magniez & Panitvong, 2005).

Habitats and ecology. All specimens of *S. angiangensis* sp. nov. were found in springs or hyporheic habitats along the banks of small mountainous rivers, located on forested slopes (see Fig. 6a). These crustaceans appear to inhabit the lower river substrate layer composed of pebbles and sand, where moisture is retained even during the prolonged dry season with no rain (lasting 3–4 months). No specimens were found in cryptic microhabitats such as beneath large rocks or within the main river channel, suggesting a preference for stable, humid interstitial environments near stream banks.

Distribution. The species is known only from the isolated mountainous massifs (free-standing hills), located in the western part of An Giang Province, which is part of the Mekong River Delta region in southern Vietnam.

ZooBank taxon ID. The electronic version of this article in Portable Document Format will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank Life Science Identifiers (LSID) for this publication is: https://zoobank.org/8F6D2242-2FE4-49A4-949C-56A75C39DA6F. The online version of this work is archived and available from the following digital repositories: ZooKeys, PubMed Central and CLOCKSS. The LSID for the new species, *Stenasellus angiangensis* Marin, Palatov, Thinh, Douady & Malard, sp. nov., is https://zoobank.org/997DB87C-7A52-4B3C-932F-BDCD2760A306.

Discussion

Our updated version of the phylogeny of Stenasellidae provides new knowledge and significantly expands the latest version presented by Jugovic et al. (2024). First, we documented the existence of a well-supported and geographically widespread Asian clade comprising *Stenasellus* species from Iran (*S. tashanicus* and *S. stygopersicus*), India (species awaiting formal description, this study), Cambodia (*S. cambodianus*) and southern Vietnam (*S. cambodianus* and *S. angiangensis* sp. nov.). Magniez (2005) suggested that *Stenasellus* might have dispersed along major river systems of mainland Southeast Asia flowing from the eastern Himalaya, though he had not predicted the presence of that genus in India. Identifying the southern limits of that Asian clade in Southeast Asia would require sequencing *Stenasellus* species described from Malaysia, Sumatra, Borneo and Java. Second, as part of the present study, we successfully developed new PCR primers for amplifying the FASTKD4 gene in four MOTUs of *Metastenasellus*

from Western and Middle Africa (Suppl. material 1: table S1), thereby enabling this genus to be included for the first time in the phylogeny of Stenasellidae. Third, our phylogenetic reconstruction identified six well-supported polytypic clades within the Stenasellidae and two monotypic clades. These clades have non overlapping geographic distributions: the genus *Metastenasellus* is present in Africa (clade 1), the genera *Balkanostenasellus* and *Protelsonia* are found in the Balkans (clade 5), and the four clades identified within the genus *Stenasellus* occupy the southern Iberian Peninsula for clade 3, southern France, Corsica, northern Spain, Tuscany (Italy), and northern Sardinia for clade 4, southern Sardinia for clade 6, and a large part of Asia stretching from Iran to Vietnam for clade 2. However, relationships among the eight clades remain unresolved, limiting our ability to reconstruct deep evolutionary events within the group.

Despite new phylogenetic knowledge acquired on the Stenasellidae, key hypotheses regarding the origin and biogeographic history of that family remain untested. To explain the present-day distribution of stenasellids, Magniez (1999) hypothesized that marine ancestors had colonized continental groundwater systems from coastal sediments of the Neo-Tethys Sea, no later than during the Upper Cretaceous. Based on morphological characters, he further proposed two independent colonization events: one giving rise to *Stenasellus* and *Acanthastenasellus*, and the other to an "Atlantic group" of taxa, including all other stenasellid genera from West Africa, America and the Balkans (herein, *Balkanostenasellus*, *Magniezia*, *Metastenasellus*, and *Protelsonia*) (Malard et al. 2014). However, our phylogeny does not support such a colonization scenario since it does not support the monophyly of either the "Atlantic group" or that of the genus *Stenasellus*. That said, we cannot reject that scenario with certainty because phylogenetic relationships among clades are not resolved. Species belonging to the Asian clade (clade 2) exhibit the morphological diagnostic features of the genus *Stenasellus*, in that their male pleopod II all possess a biarticulated endopodite with a fusiform distal segment of variable shape (Magniez 1966). Species of *Metastenasellus* differ morphologically from species of *Stenasellus* in that the endopodite of their male II pleopod is secondarily unarticulated, spiraled in its apical part, and contains a helicoidal spermatic canal (Magniez 1979; Zébazé Togouet et al. 2013; Pountougnigni et al. 2021). *S. nuragicus* and *S. assorgiai* (clade 6, southern Sardinia) also exhibit the morphological features that are diagnostic of the genus *Stenasellus*. However, when describing these two species, Argano (1968) suggested that they probably belonged to a different clade to clade 3 (the *Stenasellus galhanoe* group at that time) and clade 4 (the *Stenasellus virei* group at that time). A critical next step for evaluating Magniez's (1999) scenario of stenasellid biogeography is to incorporate American genera (i.e. *Mexistenasellus* and *Etlastenasellus*) and west African stenasellids (i.e. *Stenasellus* and *Acanthastenasellus*) into a global phylogeny. This would allow for a more complete reconstruction of the evolutionary history of Stenasellidae.

Historically, the taxonomy of Stenasellidae has long been based solely on morphological characters (i.e. morphospecies; Magniez 1991). Recent taxonomic studies used molecular methods, either in isolation or in combination with morphological methods, to determine species boundaries (Pountougnigni et al. 2021; Jugovic et al.

2024; Lagnika et al. 2024). They revealed that selected morphospecies of stenasellids were generally heterogeneous genetically, often encompassing multiple MOTUs as delimited through various COI-based molecular species delimitation methods (Morvan et al. 2013; Eme et al. 2018; Jugovic et al. 2024; Saclier et al. 2024). These methods differ in their criteria for identifying species boundaries, leading to inevitable variation in the number of MOTUs they delineate (Dellicour and Flot 2015, 2018; Puillandre et al. 2021). Moreover, COI-based species delimitation methods can overestimate the richness of putative species compared to genome-wide, nuclear-based delimitation approaches (Hupało et al. 2023). To mitigate the risk of taxonomic over splitting due the sole use of COI-based species delimitation methods, we adopted a conservative strategy for defining species within the Asian clade. We assumed that specimens from different populations (i.e. sites) could be assigned to a single nominal species if at least one delimitation method groups these populations within a single MOTU. Both the TH and mPTP methods grouped all populations of *Stenasellus angiangensis* sp. nov. within a single MOTU and mPTP also grouped the two populations of *S. cambodianus* within a single MOTU. Second, several MOTUs defined by a molecular species delimitation method could be grouped under the same nominal species if they formed a clade and their specimens shared consistent and distinct morphological characteristics that distinguished them from other Stenasellidae. The ASAP, ABGD, PTP, and bPTP methods all distinguished three MOTUs within *Stenasellus angiangensis* sp. nov. All processed specimens from these three MOTUs shared distinctive morphological characteristics, most notably a male endopod II terminating in a strong spur and a flute-shaped efferent canal (see Fig. 10f), a unique morphological feature not found in any other species of stenasellids, including its sister species, *S. cambodianus*. The latter comprised specimens from two localities, one in Cambodia (type locality) and the other in Vietnam, corresponding to distinct but sister MOTUs as delimited by all six delimitation methods, except mPTP. All specimens processed from these two MOTUs shared a male endopod II that also ended in a strong spur, as in *Stenasellus angiangensis* sp. nov., but the efferent canal was narrow (Boutin and Magniez 1985) rather than flute-shaped (Fig. 10f). The species status of *Stenasellus angiangensis* sp. nov. and *S. cambodianus* was corroborated by the 28S gene, which revealed a much greater evolutionary divergence between populations of these two species than within each species.

It is tempting to interpret the high intraspecific COI divergence observed in *Stenasellus angiangensis* sp. nov. and *S. cambodianus* as the consequence of the geographical isolation of their populations in separate mountainous hills (islands) during periods of rising sea levels. For example, the mountainous areas of the southern Mekong River Delta were likely fragmented into isolated highland islands during the late Messinian–early Pliocene interval (approximately 6 to 3.6 Mya), a period characterized by high sea levels, up to 50 meters above present levels (see fig. 3 in Woodruff (2003)). However, other factors besides sea level fluctuations could have contributed to divergence among geographically close populations, as even populations located on the same island exhibit substantial COI-divergence (i.e. COI sequences of populations of *S. angiangensis* sp. nov. from sites 10 and 11 diverged by 6.1%). Such a strong genetic structuring on a relatively small spatial scale is not unique in stenasellids: similar patterns have been observed in the

Cantabrian Range, Spain (11 MOTUs; Jugovic et al. 2024) and the Ouémé catchment, Benin (11 MOTUs, Lagnika et al. 2024), suggesting the capacity for localized speciation. Accordingly, we anticipate that further continued sampling in the Mekong River catchment will reveal additional, currently undescribed *Stenasellus* species.

Acknowledgements

The study was carried out within the framework of the program E-3.4 "The ecosystem of the Mekong River in the context of global climate change and anthropogenic impact" of the Joint Vietnam–Russia Tropical Science and Technology Research Center (Ho Chi Minh City, Vietnam) and, in particular, Task 7 "Study of biodiversity and ecosystem structure of coastal and floodplain territories ". The study was supported by the French National Research Agency under project EUR H20'Lyon (ANR-17-EURE-0018) and NeGA (ANR-20-CE02-0008), Biodiversa+, the European Biodiversity Partnership under the 2021–2022 BiodivProtect joint call for research proposals, co-funded by the European Commission (Project DarCo, GA no. 101052342), IRP CNRS (Project: 'Effects of River-Aquifer Exchanges on Riverine Ecosystem Resilience to Global Change'), and IUCN Vietnam (project number P01221, 2014: 'Baseline survey of biodiversity in Kien Giang Province, Vietnam'). Authors are deeply grateful to the administration and stuff of the Southern Department of the Joint Vietnam–Russia Tropical Science and Technology Research Center (Ho Chi Minh City, Vietnam), especially to the head of the Laboratory of Hydrobiology Dr. Nguyên Cù Định. We are sending our special thanks to Roman A. Rakitov (Paleontological Institute of RAS) for his help with obtaining SEM images, and Trần Văn Tién (Tropical Centre, Ho Chi Minh City) for help during the field sampling in Vietnam. We thank Dr. Luu Hong Truong (Academy of Science and Technology, Ho Chi Minh City, Vietnam) for organizing sampling in Hang Giếng Tiên Cave (Vietnam) and Shaniyaz Menliev and the Ministry for Agriculture and Environment Protection of Turkmenistan for providing access to sampling sites in Turkmenistan. We thank the following persons for collecting samples: Josiane Lips, Bernard Lips, and Jozef Grego (sampling sites in Turkmenistan), Anne Bedos (Kampong Trach water cave, Cambodia), Marko Lukic (Hang Giếng Tiên Cave, Vietnam), and Florian Mermillod-Blondin (sampling site in India). We thank Colin Issartel at CNRS – Université Lyon 1 for his help in identifying and dissecting stenasellids. We thank the associate editor and one anonymous reviewer for comments on an earlier draft of this manuscript.

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Supplementary material 1

Supplementary tables

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Data type: docx

Explanation note: **table S1.** List of primers. **table S2.** Alignment of the COI sequences used for the delimitation of MOTUs. **table S3.** Metadata for the COI sequences used for the delimitation of MOTUs. **table S4.** Alignment of the 16S sequences used for the four-gene phylogeny. **table S5.** Alignment of the COI sequences used for the four-gene phylogeny. **table S6.** Alignment of the FASTKD4 sequences used for the four-gene phylogeny. **table S7.** Alignment of the 28S sequences used for the four-gene phylogeny. **table S8.** Metadata for DNA sequences used for the four-gene phylogeny. **table S9.** Genetic distances between and within populations of Stenasellidae collected in Cambodia and Vietnam. **table S10.** Metadata for the DNA sequences deposited in NCBI as part of this study.

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Link: https://doi.org/10.3897/subtbiol.55.185185.suppl1